J. Pharm. Pharmacol. 1980, 32: 773–777 Received July 14, 1980

# A sensitive benzodiazepine radioimmunoassay of broad specificity

KATHERINE ROBINSON, MARY G. RUTTERFORD AND R. N. SMITH*

*Metropolitan Police Forensic Science Laboratory, 109 Lambeth Road, London SE1 7LP, U.K.*

Four benzodiazepine radioimmunoassays are described, the most sensitive of which can detect sub-therapeutic levels of bromazepam, chlordiazepoxide, clobazam, demoxepam, desalkylflurazepam, desmethyldiazepam, diazepam, flunitrazepam, lorazepam, medazepam, nitrazepam, oxazepam, prazepam, temazepam and triazolam. The standard curve for diazepam has a concentration range of 0–2·5 ng $ml^{-1}$. The assay is particularly applicable to blood samples of forensic interest that may be haemolysed or decomposing. A 75 $\mu$l sample is required. The antiserum and [$^3$H]flunitrazepam used are commercially available.

The convenience of radioimmunoassay (RIA) for analysing diazepam in small blood samples of forensic interest (Rutterford & Smith 1980) prompted the development of a more general assay capable of detecting a number of commonly-prescribed benzodiazepines. For this, an antiserum with a broad spectrum of cross-reactivity was required. Initially we used the antiserum from an Emit-dau benzodiazepine metabolite kit with [$^3$H]diazepam and devised an assay (Assay 1) that could detect therapeutic or sub-therapetuc levels of chlordiazepoxide, clobazam, demoxepam, desalkylflurazepam, desmethyldiazepam, diazepam, nitrazepam, oxazepam, prazepam and temazepam. Medazepam could be detected as its metabolite, diazepam, while lorazepam, a widely-prescribed drug, could only be detected at high therapeutic or overdose levels. Bromazepam, clonazepam, flunitrazepam and triazolam cross-reacted poorly and could not be assayed satisfactorily. Assay 1 proved very useful in forensic casework for detecting commonly-encountered benzodiazepines, but a more sensitive assay was required for cases of suspected lorazepam ingestion. Attempts to increase the sensitivity of Assay 1 by varying the reagent concentrations and by using sequential saturation techniques failed, and so the antiserum from the recently-available Emit-tox serum benzodiazepine assay kit was tested using [$^3$H]diazepam as the label (Assay 2). More sensitive standard curves resulted for many benzodiazepines with the exception of chlordiazepoxide, clobazam, demoxepam, lorazepam and oxazepam. [$^3$H]Flunitrazepam was then tried with both the Emit-dau and Emit-tox antisera (Assays 3 and 4 respectively) to determine whether, since flunitrazepam is less strongly bound by the antisera than diazepam, more sensitive standard curves would result. Assay 3 gave, in general, relatively insensitive curves but, with Assay 4, all the benzodiazepines tested with the exception of clonazepam could be detected at sub-therapeutic levels. Assay 4 was therefore investigated in detail and has replaced Assay 1 in forensic casework. The assay is simple and economical, and has the advantage over many published RIA methods for drugs that the reagents are commercially available. It requires only 75 $\mu$l of blood which may be haemolysed or decomposing.

* Correspondence.

## MATERIALS AND METHODS

The rationale dictating the choice of assay conditions resembles that of the specific diazepam assay referred to in the introduction.

### *Buffer*

0·067 M phosphate of pH 7·4 containing 0·2% bovine $\gamma$-globulin (Cohn Fraction II, Sigma Chemical Co., Fancy Road, Poole, Dorset, U.K) and 0·1% sodium azide.

### *Antisera*

Emit-dau benzodiazepine metabolite and Emit-tox serum benzodiazepine assay kits are purchased from Syva, St. Ives House, Maidenhead, Berks., U.K. The antiserum in the Emit-dau kits is in solution while that in the Emit-tox kits is supplied freeze-dried and requires reconstitution with 3 ml distilled water. Both antisera are diluted tenfold with buffer and stored at −20 °C in 100 $\mu$l aliquots. For an assay, an aliquot is allowed to thaw and buffer is

added to give the dilution required to bind 50–60% of the tritiated benzodiazepine added to each assay tube. Alternatively, the antisera may be stored at 2–4 °C and portions diluted with buffer as required. There is sufficient antiserum in a 50-tube Emit-tox kit to prepare over 20 000 RIA tubes.

*[$^3$H]diazepam and [$^3$H]flunitrazepam*

[*N-methyl*-$^3$H]diazepam and [*N-methyl*-$^3$H]flunitrazepam (60–85 Ci mmol$^{-1}$; 2·2–3·1 TBq mmol$^{-1}$) are purchased from the Radiochemical Centre, Amersham, Bucks., U.K. Stock solutions of 50 $\mu$Ci in 3·5 ml ethanol are stored at −20°C in polypropylene tubes. For an assay, 50 $\mu$l of stock solution are diluted to 5 ml with buffer.

*Benzodiazepine standards*

These are stored in aliquots of any convenient concentration at −20 °C in silanized glass vials. For an assay, an aliquot is thawed and diluted with buffer to give the required range of standards. The zero standard is buffer.

*Sample preparation*

75 $\mu$l of blood are buffered to about pH 9 and vortexed for 30 s with 500 $\mu$l ethyl acetate. After centrifugation (1 min, 12 000 *g*), 400 $\mu$l of the ethyl acetate extract are evaporated to dryness in a polypropylene tube under compressed air at room temperature (20 °C). 150 $\mu$l of buffer are added to the residue to give a 1:2·5 dilution of the original sample. 25 $\mu$l of this solution are diluted to 1:25 with buffer, and an aliquot of this is further diluted to 1:250. A 1:2500 dilution may also be required if high levels of the more cross-reactive benzodiazepines are to lie within the assay range.

Initially, samples were deproteinized with MeOH and, after centrifugation, an aliquot of the methanol extract was evaporated to dryness and reconstituted in buffer. This procedure, however, was found to produce false positive results in Assay 1 when blank samples were assayed, presumably due to the co-extraction of some constituent of the samples that bound to the antiserum. The problem was avoided by the ethyl acetate extraction described above. Other solvents were also tried, but only ethyl acetate was reasonably convenient to pipette, eliminated false positive results and extracted all the benzodiazepines tested in good yield.

*PEG solution*

23% w/v polyethylene glycol (PEG) of molecular weight 6000 in buffer containing no $\gamma$-globulin.

*Scintillant*

4 g 2,5-diphenyloxazole (PPO) and 0·2 g 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) per litre of reagent-grade toluene.

*Assay protocol*

50 $\mu$l each of sample or standard, tritiated benzodiazepine and antiserum are pipetted into duplicate sets of microcentrifuge tubes. 50 $\mu$l tritiated benzodiazepine and 100 $\mu$l buffer are put in another pair of tubes to measure the total activity per tube. All tubes are vortexed, incubated at 2 °C for 1 h and then 475 $\mu$l PEG solution at room temperature are added. The tubes are vortexed thoroughly and allowed to stand at room temperature for 5–10 min before centrifuging (2 min, 12 000 *g*). 400 $\mu$l aliquots of the supernatants are transferred to 5 ml polypropylene liquid scintillation counting tubes, 4 ml scintillant are added and the tritiated benzodiazepine is extracted into the scintillant by 10 min vigorous shaking (extraction efficiency: 96% for [$^3$H]-diazepam, 99% for [$^3$H]flunitrazepam). The tubes are then counted for 2–5 min after being left in darkness in a liquid scintillation counter for 15–30 min to allow the phases to separate and any luminescence of the samples or tubes to decay to zero.

An alternative but less efficient and more expensive counting procedure is to use a 1,4-dioxane-based scintillant (e.g. Bray 1960) with which PEG solutions are miscible.

## RESULTS AND DISCUSSION

In general, RIA methods used for "drug-screening" in forensic toxicology should be sensitive, directed at classes of chemically similar drugs rather than individual compounds, and applicable to samples of whole blood that may be haemolysed or decomposing. The limiting factor in a toxicological analysis is often the amount of sample available and so as little sample as possible should be used for RIA leaving sufficient for positive results to be confirmed by other methods.

Most published RIA or radioreceptor methods for benzodiazepines (Peskar & Spector 1973; Dixon et al 1975; Dixon & Crews 1977; Dixon et al 1977; Gelbke et al 1977; Ko et al 1977; Bourne et al 1978; Hunt 1979; Hunt et al 1979; Owen et al 1979; Skolnick et al 1979) are intended for monitoring specific compounds in serum or plasma for clinical purposes and are thus of limited application in forensic work. In contrast, the assays described here were intended to detect a range of benzodiazepines

in order that one analysis would indicate whether or not further work on the sample would be justified.

The Emit antisera were found to cross-react with a surprisingly diverse range of benzodiazepines but, compared with RIA, the Emit enzyme immunoassays lack sensitivity and cannot be applied to haemolysed blood samples unless a concentrated extract is prepared (Slightom 1978). This procedure, however, is inapplicable to many of the samples submitted to this laboratory for general drug analysis since they consist of only 1–2 ml of haemolysed blood, and so RIA is preferred.

The four assays that were developed are compared in Table 1 which gives the benzodiazepine concentrations required to reduce the initial binding by 50%. Assays 2 and 3, which were no more useful than Assay 1, were not investigated further while Assay 4 was studied in detail.

The standard curves of a wide range of benzodiazepines using Assay 4 are shown in Fig. 1. Blank blood extracts at a 1:2·5 dilution give an average response of 82·4% $B/B_0$ (where B is the bound activity and $B_0$ is the activity bound in the absence of unlabelled drug) with a standard deviation of 7·1% $B/B_0$ ($n = 31$). The horizontal line in Fig. 1 at the 68·2% $B/B_0$ level corresponds to the mean blank response plus two standard deviations. The 50% $B/B_0$ level passes through the "useful" part of all the curves in Fig. 1, and blood extracts "spiked" with lorazepam to give a response of about 50% $B/B_0$ were used to determine the standard deviation of the assay which was found to be 4·9% $B/B_0$ ($n = 22$). The 60% $B/B_0$ line in Fig. 1 therefore corresponds to easily-measured benzodiazepine concentrations plus two standard deviations and is well separated from the "background" response of blank blood extracts.

The usefulness of the assay in practice is evaluated by calculating the dilutions at which therapeutic blood levels of the various benzodiazepines would give 50% $B/B_0$ (Table 2). The dilutions range from 1 for clonazepam to 400 for diazepam and, with the exception of clonazepam and flurazepam, subtherapeutic levels of all the benzodiazepines tested would be detected in a blood extract at the initial dilution of 1:2·5. Clonazepam, which is not widely used in the U.K., would only be detected at about three times its therapeutic level but flurazepam is easily detected as its metabolite, desalkylflurazepam. In general, Assay 4 is much more sensitive than its predecessor, Assay 1, while the background response of blank blood extracts and the standard deviation of spiked blood extracts are of the same order. The original aim of detecting therapeutic or lower levels of lorazepam is achieved and even triazolam, a relatively new benzodiazepine with a low therapeutic index, can be detected at sub-therapeutic levels.

As with any RIA displaying a broad spectrum of cross-reactivity, the interpretation of the results requires care. A positive result is the sum of the sample background and the assay response to cross-reacting species in the sample. The background decreases with increasing dilution but, at the initial dilution of 1:2·5, may contribute significantly to the result. A benzodiazepine known to be present in the sample can be used as the assay standard, but

Table 1. Benzodiazepine concentrations (ng $ml^{-1}$) required to displace 50% of bound activity.

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Antiserum | Emit-dau | Emit-tox | Emit-dau | Emit-tox |
| (dilution) | (1 : 200) | (1 : 400) | (1 : 40) | (1 : 350) |
| Label | [$^3$H]diazepam | | [$^3$H]flunitrazepam | |
| Bromazepam | >500 | 20 | 85 | 20 |
| Chordiazepoxide | 87 | 490 | 68 | 41·5 |
| Clobazam | 24 | 28 | 40 | 12·6 |
| Clonazepam | >500 | 113 | >500 | 53 |
| Demoxepam | 41 | 175 | 20 | 34·2 |
| Desalkylflurazepam | 19 | 11·5 | 27 | 1 |
| Desmethyldiazepam | 11 | 2·4 | 12·6 | 0·8 |
| Diazepam | 3·3 | 0·8 | 27 | 0·6 |
| Flunitrazepam | >500 | 43 | 12 | 1·4 |
| Flurazepam | >500 | 9 | >50 | 2·2 |
| Lorazepam | 375 | 500 | 275 | 43·5 |
| Medazepam | 50 | 39 | 100 | 6 |
| Nitrazepam | 8 | 7·5 | 10·5 | 1·3 |
| Oxazepam | 2·5 | 16 | 26 | 6·3 |
| Prazepam | 30 | 6 | >100 | 5·2 |
| Temazepam | 19 | 16 | 35 | 1·5 |
| Triazolam | >500 | 190 | >500 | 4·8 |

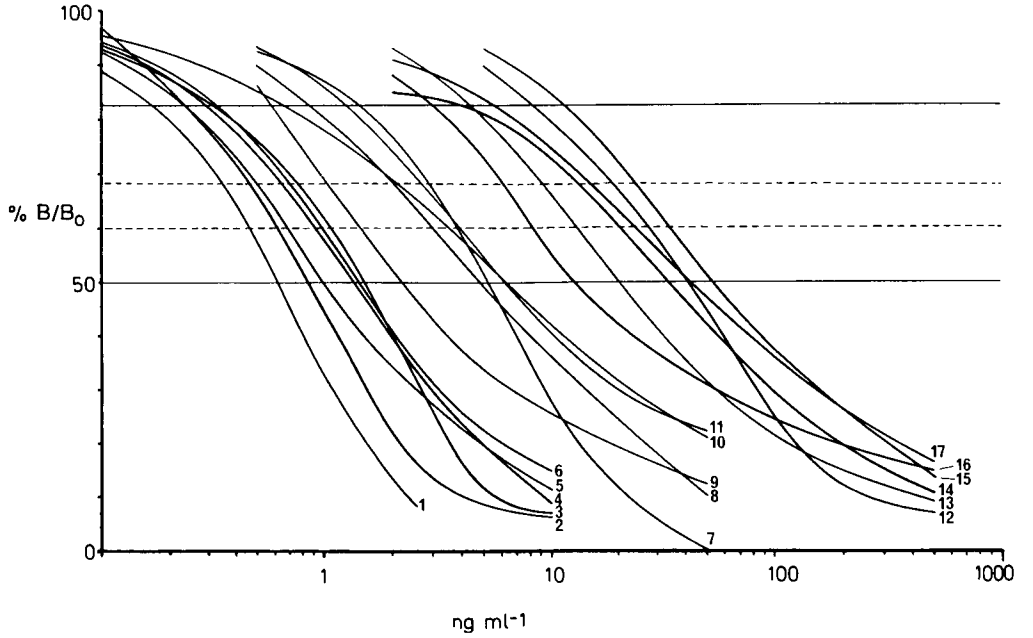


FIG. 1. Standard curves of benzodiazepines dissolved in buffer. B = bound activity; $B_0$ = activity bound in absence of unlabelled drug. 1, Diazepam; 2, Desmethyldiazepam; 3, Temazepam; 4, Flunitrazepam; 5, Desalkylflurazepam; 6, Nitrazepam; 7, Prazepam; 8, Triazolam; 9, Flurazepam; 10, Oxazepam; 11, Medazepam; 12, Chlordiazepoxide; 13, Bromazepam; 14, Demoxepam; 15, Clonazepam; 16, Clobazam; 17, Lorazepam.

Table 2. Evaluation of Assay 4 and comparison with Assay 1.

| | Approximate average therapeutic blood level (ng $ml^{-1}$) | Approximate dilution factor required for average therapeutic blood level to give 50% $B/B_0$ | |
|---|---|---|---|
| | | Assay 1 | Assay 4 |
| Bromazepam | 300 (1)* | —† | 15 |
| Chlordiazepoxide | 350 (2) | 4 | 8 |
| Clobazam | 500 (3) | 21 | 40 |
| Clonazepam | 50 (4) | —† | 1 |
| Demoxepam | 350 (5) | 4 | 40 |
| Desalkylflurazepam (after flurazepam ingestion) | 75 (6) | 4 | 75 |
| Desmethyldiazepam (after diazepam ingestion) | 250 (7,8) | 23 | 300 |
| Diazepam | 250 (7, 8) | 75 | 400 |
| Flunitrazepam | 50 (9) | —† | 37 |
| Lorazepam | 150 (10) | 2·5 × conc. | 3·5 |
| Medazepam | 100 (11) | 2 | 16 |
| Nitrazepam | 40 (11) | 5 | 30 |
| Oxazepam (after diazepam ingestion) | 100 (8, 12) | 40 | 16 |
| Prazepam | 2000 (13) | 67 | 385 |
| Temazepam (after diazepam ingestion) | 75 (8) | 4 | 50 |
| Triazolam | 25 (14) | —† | 5 |

* Numbers in parentheses refer to publications cited below.

† Standard curve levels out above 50% $B/B_0$.

1, de Silva & Kaplan 1966; 2, Greenblatt et al 1976; 3, Rupp et al 1979; 4, Sjö et al 1975; 5, Chlordiazepoxide metabolite. Blood level approximately that of chlordiazepoxide (Bond et al 1977); 6, Hasegawa & Matsubara 1975; 7, Garattini et al 1973; 8, Zingales 1973; 9, Boxenbaum et al 1978; 10, Kyriakopoulos 1976; 11, Greenblatt & Shader 1974; 12, Knowles & Ruelius 1972; 13, Vesell et al 1972; 14, Ko et al 1977.

the presence of metabolites that may cross react more or less than the parent compound means that the result is likely to be an over-estimate and is best considered semi-quantitative even when the dilution is high enough to eliminate the sample background. This detracts little from the value of the assay as a "screening" technique since it is customary in forensic toxicology to confirm positive results by alternative methods. Indeed, the ability to detect metabolites increases the sensitivity of the assay in, for instance, cases of continuous diazepam therapy where the metabolite levels can greatly exceed those of the parent compound (Zingales 1973).

No absolute "cut-off" value can be defined but a response of about 60% $B/B_0$ or lower can be considered positive while a higher response may be due to the sample background either alone or combined with a low benzodiazepine level. Usually there is little difficulty in deciding which samples require further investigation since a response of 60% $B/B_0$ from a blood extract at a dilution of 1:2·5 corresponds to a sub-therapeutic level of any benzodiazepine tested except clonazepam. Even in the case of lorazepam, relatively low levels may be detected. For example, two blood extracts at a dilution of 1:2·5 gave $\%B/B_0$ values of 58·6 and 67·4, corresponding to lorazepam blood levels of 68 and 40 ng $ml^{-1}$ respectively, while gas chromatography gave levels of 56 and 28 ng $ml^{-1}$.

## REFERENCES


Bond, A. J., Hailey, D. M., Lader, M. H. (1977) Br. J. Clin. Pharmacol. 4: 51–56

Bourne, R. C., Robinson, J. D., Teale, D. J. (1978) Br. J. Pharmacol. 63: 371

Boxenbaum, H. G., Posmanter, H. N., Macasieb, T., Geitner, K. A., Weinfeld, R. E., Moore, J. D., Darragh, A., O'Kelly, D. A., Weissman, L., Kaplan, S. A. (1978) J. Pharmacokinet. Biopharm. 6: 283–293

Bray, G. A. (1960) Anal. Biochem. 1: 279–285

De Silva, J. A. F., Kaplan, J. (1966) J. Pharm. Sci. 55: 1278–1283

Dixon, R., Crews, T. (1977) Res. Comm. Chem. Pathol. Pharmacol. 18: 477–486

Dixon, W. R., Earley, J., Postma, E. (1975) J. Pharm. Sci. 64: 937–939

Dixon, W. R., Young, R. L., Ning, R., Liebman, A. (1977) Ibid. 66: 235–237

Garattini, S., Marcucci, F., Marselli, P. L., Mussini, E. (1973) in: Davies, D. S., Prichard, B. N. C. (eds) Biological Effects of Drugs in relation to their Plasma Concentrations. MacMillan Press Ltd., London, pp 211–225

Gelbke, H. P., Schlicht, H. J., Schmidt, G. (1977) Arch. Toxicol. 38: 295–305

Greenblatt, D. J., Shader, R. I. (1974) Benzodiazepines in Clinical Practice. Raven Press, New York, pp 17–42

Greenblatt, D. J., Shader, R. I., Koch-Weser, J., Franke, K. (1976) in: Gottschalk, L. A., Merlis, S. (eds) Pharmacokinetics of Psychoactive Drugs: Blood Levels and Clinical Response. Spectrum Publications, New York, pp 127–139

Hasegawa, M., Matsubara, I. (1975) Chem. Pharm. Bull. 23: 1826–1833

Hunt, P. (1979) Br. J. Clin. Pharmacol. 7: 37S–40S

Hunt, P., Husson, J.-M., Raynaud, J.-P. (1979) J. Pharm. Pharmacol. 31: 448–451

Knowles, J. A., Ruelius, H. W. (1972) Arzneim.-Forsch. 22: 687–692

Ko, H., Royer, M. E., Hester, J. B., Johnston, K. T. (1977) Anal. Lett. 10: 1019–1040

Kyriakopoulos, A. A. (1976) in: Gottschalk, L. A., Merlis, S. (eds) Pharmacokinetics of Psychoactive Drugs: Blood Levels and Clinical Response. Spectrum Publications, New York, pp 45–60

Owen, F., Lofthouse, R., Bourne, R. C. (1979) Clin. Chim. Acta 93: 305–310

Peskar, B., Spector, S. (1973) J. Pharm. Exp. Ther. 186: 167–172

Rupp, W., Badian, M., Christ, O., Hajdú, P., Kulkarni, R. D., Taeuber, K., Uihlein, M., Bender, R., Vanderbeke, O. (1979) Br. J. Clin. Pharmacol. 7: 51S–57S

Rutterford, M. G., Smith, R. N. (1980) J. Pharm. Pharmacol. 32: 449–452

Sjö, O., Hvidberg, E. F., Naestoft, J., Lund, M. (1975) Eur. J. Clin. Pharmacol. 8: 249–254

Skolnick, P., Goodwin, F. K., Steven, M. P. (1979) Arch. Gen. Psychiatry 36: 78–80

Slightom, E. L. (1978) J. Forensic Sci. 23: 292–303

Vesell, E. S., Passananti, G. T., Viau, J.-P., Epps, J. E., Di Carlo, F. T. (1972) Pharmacology 7: 197–206

Zingales, I. A. (1973) J. Chromatogr. 75: 55–78